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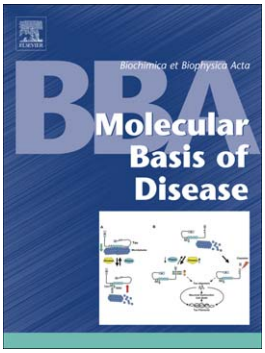

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Caenorhabditis elegans, a pluricellular model organism to screen new genes involved in mitochondrial genome maintenance.

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Summary

The inheritance of functional mitochondria depends on faithful replication and transmission of mitochondrial DNA (mtDNA). A large and heterogeneous group of human disorders is associated with mitochondrial genome quantitative and qualitative anomalies. Several nuclear genes have been shown to account for these severe OXPHOS disorders. However, in several cases the disease-causing mutations still remain unknown.

C. elegans has been largely used for studying various biological functions as this multicellular organism has short life cycle and is easy to grow in the laboratory. Mitochondrial functions are relatively well conserved between human and *C. elegans* and heteroplasmy exists in this organism as in human. *C. elegans* therefore represent a useful tool for studying mtDNA maintenance. Suppression by RNA interference of genes involved in mtDNA replication such as *polg-1*, encoding the mitochondrial DNA polymerase, results in reduced mtDNA copy number but in a normal phenotype of the F1 worms. By combining RNAi of genes involved in mtDNA maintenance and EtBr exposure we were able to reveal a strong and specific phenotype (developmental larval arrest) associated to a severe decrease of mtDNA copy number. Moreover, we tested and validated the screen efficiency for human orthologous genes encoding mitochondrial nucleoid proteins. This allowed us to identify several genes that seem to be closely related to mtDNA maintenance in *C. elegans*.

This work reports a first step in the further development of a large-scale screening in *C. elegans* that should allow to identify new genes of mtDNA maintenance whose human orthologs will obviously constitute new candidate genes for patients with quantitative or qualitative mtDNA anomalies.

Introduction

Mitochondria play an essential role in cellular energy production provided by the mitochondrial respiratory chain. The inheritance of functional mitochondria depends on faithful replication and transmission of mitochondrial DNA (mtDNA). The mitochondrial genome encodes only a few proteins of the respiratory chain (RC) complexes whereas all other mitochondrial proteins are encoded in the nucleus, synthesized on cytosolic ribosomes and imported into mitochondria. The mitochondrially encoded proteins are essential for the proper function of the respiratory chain and expression of those subunits requires that the mitochondrial genome be replicated, transcribed and translated. The newly synthesized subunits are subsequently assembled with nuclear encoded subunits. Over the past years, while key details of the content and expression of the mitochondrial genome have been elucidated, important questions remain regarding the maintenance and the transmission of mtDNA during cell proliferation and development.

A large and heterogeneous group of disorders is associated with mitochondrial genome anomalies in human. Mutations in nuclear genes encoding proteins involved in mtDNA maintenance can result in large-scale mtDNA rearrangements (mtDNA deletion) and abnormal copy number (mtDNA depletion) of the mitochondrial genome. Autosomal dominant progressive external ophthalmoplegia (adPEO) is an adult-onset mitochondrial disorder characterized by ophthalmoparesis with exercise intolerance, ataxia, peripheral neuropathy, and multiple mtDNA deletions [1, 2]. Familial ad-PEO is genetically heterogeneous, and at least six nuclear genes account for this disease, namely, *ANT1,* encoding the adenosine diphosphate-triphosphate translocator [3, 4], *POLG1* [5] and *POLG2* [6] encoding the catalytic and accessory subunits of the mtDNA polymerase, *PEO1* encoding the Twinkle DNA helicase [1], *OPA1* encoding a mitochondrial dynamin-related GTPase [7] and *RRM2B* encoding the p53-inducible small subunit of ribonucleotide reductase [8].

mtDNA depletion syndrome (MDS) is a clinically and genetically heterogeneous group of autosomal recessive diseases characterized by a reduction in mtDNA copy number [9]. Several nuclear genes have been shown to account for these severe OXPHOS disorders. Indeed, mutations in the deoxyguanosine kinase (*DGUOK*) and the thymidine kinase genes (*TK2*) have been reported in the hepatocerebral and myopathic forms of MDS [10, 11] respectively. Also, *POLG1* mutations associated with Alpers' syndrome [12, 13] as well as the hepatocerebral form lead to mtDNA depletion. Mutations in TP gene encoding the cytosolic thymidine phosphorylase result in mitochondrial neurogastrointestinal encephalopathy (MNGIE) syndrome with mtDNA deletion and depletion [14]. The succinyl coenzyme A-ligase subunits (*SUCLA2,* [15] and SUCLG1 [16]) and *MPV17* [17] genes have

also been shown to account for mtDNA depletion in a few pedigrees. Finally, mutations of *RRM2B* encoding a small subunit of the cytosolic ribonucleotide reductase have been reported to cause severe muscle mtDNA depletion [18]. All these genes are involved in mtDNA maintenance or dNTP metabolism. However, in several cases of ad-PEO and MDS the disease-causing mutations remain still unknown.

The suitability of the yeast *Saccharomyces cerevisiae* as a model for human mitochondrial studies has been well demonstrated. However notable differences in mtDNA structure and dynamics between yeast and human did not make it a perfect tool to study the mtDNA maintenance. Indeed, human cells contain10^2–10^4 mtDNA copies whereas yeast cells contain only 20–100 copies. The mitochondrial genome, 16.6 kb in human and 85.8 kb in yeast, is predominantly linear in yeast but is circular in human [19-21]. Finally heteroplasmy is very frequently observed for mtDNA mutations in human whereas yeast cannot normally maintain stably heteroplasmy [22, 23]. Furthermore, because this yeast can grow robustly by fermentation in the absence of mtDNA, it loses its mitochondrial genome very rapidly upon inactivation of a large class of genes encoding mitochondrial proteins involved in almost all the mitochondrial biogenesis pathways (mitochondrial translation, ATP synthesis, iron homeostasis, mitochondrial import and morphology). As such it cannot be used easily to address the question of mtDNA transmission control [24].

The characteristics of *Caenorhabditis elegans* make it a perfect complement to the yeast system. While *C. elegans* has been largely used for studying various biological functions such as neuron development or apoptosis, few studies have focused on mitochondria. Its mitochondrial genome (13.7 kb) has been fully sequenced, it encodes 12 mitochondrial RC subunits and is similar in size and gene content to its human counterpart [25]. The group of B. Lemire has brought a large contribution by showing that heteroplasmy exists in this organism as in human as far as mtDNA deletions are considered [26]. This team also showed that mtDNA copy number is coordinated with the maturation steps of *C. elegans* life cycle. Indeed, mtDNA amplification is necessary for normal development as blocking mtDNA replication results in development arrest [27]. The mtDNA copy number is strictly regulated during *C. elegans* development. Indeed, mtDNA content that is maternally derived remains essentially unchanged from embryo up to the L3 larval stage. The copy number then increases at least 3-fold in L4 larvae and is associated with somatic cells development and gonad formation. A further huge increase in mtDNA content takes place in adult hermaphrodites during oogenesis and high production of embryo [27, 28]. Finally, blockage of mtDNA replication by ethidium bromide (EtBr), a well-known molecule acting as a DNA-intercaling dye and potent inhibitor of mtDNA transcription and replication [29-31] results in developmental arrest [27]. Surprisingly, inactivation by RNA interference (RNAi)

over one generation of genes encoding proteins essential for mtDNA replication such as *polg-1* (DNA polymerase γ) or *mtss-1* (mitochondrial single-stranded DNA-binding protein) does not give rise to a strong phenotype [32-36]. Only continuous exposure to RNAi over several generations revealed an abnormal phenotype (gonad protrusion and sterility, [37]). Furthermore, homozygous *polg-1* knockout mutants had normal development rates indicating a high maternal contribution of *polg-1*. However, loss of *polg-1* leads to complete sterility and shortened lifespan. Moreover during adult life, worms failed to maintain normal mtDNA levels [28].

Several genes are known to be involved in mtDNA maintenance in various species. Not only genes encoding mitochondrial proteins but also cytosolic proteins are involved in this mechanism such as *RRM2B* and *TP* in human. This suggests that unexpected genes could be required for normal mtDNA metabolism that have to be identified. In the present study, we designed an efficient and rapid screening for the identification of genes required for mtDNA maintenance in the worm *C. elegans* by combining RNAi and EtBr exposure. Considering that mitochondrial functions are relatively well conserved during evolution, the systematic use of this screen for all *C. elegans* genes will certainly allow to identify new genes of mtDNA maintenance whose human orthologs could obviously constitute new candidate genes for patients.

Results

polg-1 and *mtss-1* knock down results in mtDNA depletion

We first silenced two genes, *polg-1* and *mtss-1* in wild-type animals. These two genes are orthologs to the human *POLG1* and *SSBP1* genes encoding the mitochondrial DNA polymerase γ and the single strand DNA binding protein respectively, both genes being involved in mtDNA replication. Synchronized N2 embryos were subjected to RNAi feeding until the adult stage and during the F1 progeny. As expected, the F1 progeny developed into adulthood with no strong phenotype (Fig. 1A). We next estimated by quantitative PCR the mtDNA copy number in F1 adults having laid all their eggs to avoid any differences due to oocyte production. The mtDNA copy number in the hermaphrodites fed by bacteria expressing *polg-1* or *mtss-1* dsRNA was approximately 80% and 70% respectively lower than the control adult hermaphrodites (Fig. 1B) but similar to that of L4 worms treated with very high concentration of EtBr (125 μg/ml), concentration already known to lead to mtDNA depletion [27].

As *POLG1* mutations in human can result in either quantitative or qualitative mtDNA anomalies, we looked for mtDNA deletions in worms subjected to *polg-1* knock-down. Long-range PCR amplification of the nearly two-thirds of the genome in these worms revealed the

same mtDNA length product (9 kb) in *polg-1* treated animals and controls ruling out that *polg-1* inactivation results in mtDNA deletion (Fig. 1C).

polg-1 silencing by RNAi was highly efficient and specific as *polg-1* transcript was severely impaired in F1 progeny when subjected to RNAi feeding (Fig. 1D). We then analyzed the efficiency of *mtss-1* RNAi *in vivo*. For this purpose, we constructed transgenic animals expressing a *mtss-1::GFP* fusion construct under the control of either the body-wall muscle specific *myo-3* promoter (myo3-MTSS::GFP) or *mtss-1* promoter and 3'-UTR regulatory sequences (par-MTSS::GFP). As shown in Figure 1E, the GFP signal appeared cytoplasmic and as punctuate foci. This signal is aligned and regularly spaced along myofibrilla within the cytoplasm of body-wall muscle cells in agreement with a mitochondrial localization. Feeding these transgenic worms with bacteria expressing *mtss-1* dsRNA led to the loss of most of the MTSS::GFP punctuated signals (Fig. 1E), showing the efficiency of RNAi. Altogether, these results show that RNAi of *polg-1* and *mtss-1* over one generation lead to a specific knock-down of *polg-1* and *mtss-1*, to a severe mtDNA depletion in somatic cells but does not result in major anomalies in worms development.

Ethidium bromide exposure combined with *polg-1* and *mtss-1* knock-down results in an obvious developmental phenotype

To design a large scale screening of genes involved in mitochondrial genome maintenance using RNAi feeding, we need a clear and obvious phenotype, appearing rapidly during the worms life.

Ethidium bromide (EtBr) is a well-known nucleic acid intercalating compound that preferentially inhibits mtDNA replication and transcription. Exposure to EtBr results in a total loss of mtDNA in mammalian cells (ρ° cells) [38-40] or diminished number of mtDNA copies in *C. elegans* [27] and Fig. 1B). Concentration and timing of the EtBr treatment during *C. elegans* development affects terminal developmental stages. Indeed, submitting L1 animals to high EtBr concentration (125 µg/ml) leads to a L3 stage arrest. Prolonged exposure to EtBr results in mtDNA depletion and L3 arrested larvae presented a 25-fold decrease of mtDNA content. Interestingly, the L3 arrest phenotype was reversible upon drug removal indicating that EtBr is not acting by causing mutations in either the mitochondrial or the nuclear genomes since these would be expected to be heritable and irreversible [27].

We reasoned that animals with low mtDNA content should be more sensitive to EtBr than animals with normal mtDNA content. Therefore silencing of genes controlling mtDNA copy number combined with EtBr exposure should rapidly reveal a defective phenotype. We therefore performed *polg-1* and *mtss-1* RNAi experiments on wild-type animals from the L1 stage to the adult stage (3 days). The adults (F0) were then allowed to hatch 100 eggs (F1

progeny) on NGM plates seeded with bacteria expressing *polg-1* or *mtss-1* dsRNA, with and without increasing concentrations of EtBr (10 to 80 μg/ml). We then analyzed the development of the F1 larvae and evaluated the concentration at which 100 % of the larvae were arrested at the L3 stage. We observed that 100% of control larvae were arrested at the L3 stage after treatment with 80 μg/ml EtBr as previously reported [27] whereas as few as 20 and 60 μg/ml EtBr were sufficient to stop development of worms fed with dsRNA for *polg-1* and *mtss-1* respectively (Fig. 2A and 2B). Concentration of 50 μg/ml of EtBr appeared to clearly discriminate genes important for mtDNA stability (90 to 100% L3 arrest) compared to the control (50% L3 arrest). To confirm that this L3 arrest phenotype is indeed related to mtDNA maintenance, we further examined silencing of *rrt-2* gene encoding the arginyl-tRNA synthetase predicted to be involved in mitochondrial translation and of *pmp-4* gene encoding a peroxisomal protein homologous to human ABCD1, which when mutated leads to X-linked adrenoleukodystrophy [41]. The F1 progeny of worms submitted to RNAi of those genes shows 50-55% L3 arrest as control worms (Fig 2C). This clearly shows that the increased sensitivity towards EtBr exposure is specific to an effect on mtDNA replication. Altogether, these results show that combining RNAi of genes involved in mtDNA maintenance and EtBr exposure reveals a strong and specific phenotype that can be further used for large-scale screening in *C. elegans*.

Identification of new *C. elegans* genes involved in mtDNA stability

In human, the nucleoid consists of 5-7 mtDNA copies associated with several proteins. The precise protein composition of nucleoid is still under debate and depends of the procedure used to isolate these proteins [42]. Recent studies identify a set of proteins associated with human mtDNA nucleoids [43, 44]. Among them, a set of 31 mtDNA cross-linked proteins constitutes the core nucleoid proteins (class I) that are supposed to be closely related to mtDNA replication and transcription. A second class of proteins (class II) is part of nucleoids but not crosslinked to mtDNA. Finally, class III proteins are not found in native nucleoids [43]. Considering the efficiency of our screen on *C. elegans*, we decided to successively inactivate the genes coding proteins of the mitochondrial nucleoid with the aim to reveal genes involved in mtDNA maintenance. The human protein sequences were used to explore the *C. elegans* genome database (*C. elegans* Sequencing Consortium 1998) by BLAST searching. Several open reading frames were identified, some of them being already known as true orthologous genes. Among those genes, we could study those that did not lead to embryonic lethality in large-scale RNAi screens and were present in the Ahringer RNAi feeding library. As a first screen, we retained 19 *C. elegans* genes homologous to 16 human mitochondrial nucleoid genes (Table 1). *ANT2* and *ANT3* were reported respectively

as class I and III nucleoid proteins [43]. As there is no direct orthology between any *C. elegans* ant gene and a particular human *ANT* gene, we decided to screen the five *C. elegans* mitochondrial ADP/ATP carrier proteins (*ant-1.1*, *ant-1.2*, *ant-1.3*, *ant-1.4* and *C47E12.2*) as previously reported [45]. One of them *ant-1.1* could not be included in our screen as its inactivation from L1 larval stage leads to sterility [45]. Moreover, due to the high sequence identities (84%) of *ant-1.3* and *ant-1.4* mRNA, RNAi of any of these two genes results in silencing of both ([45] and our results). Therefore results are presented for *ant-1.4* only. On the same way, *IMMT* human gene (Mitofilin) presents two orthologs in *C. elegans*, *immt-1* and *immt-2* that were both tested.

Using the Ahringer library feeding RNAi clones, we inactivated expression of those genes beginning from synchronized L1 larvae. After 3 days, adults were then allowed to lay around 100 eggs onto new RNAi plates with 50 µg/ml EtBr. At day 4 the percentages of L3 arrested larvae versus gravid adults were counted (Fig. 3). This revealed a first group of genes for which inactivation resulted in 85 to 100% of L3 arrest. These genes are homologs of human genes encoding proteins of class I, *mtss-1*, *polg-1*, *hmg-5*, *polrmt (Y105E8A.23)*, *dnj-10*, *ANT (C47E12.2*) and *ant-1.4* but also of class II, *atad-3*, *immt-1* and of class III, *phi-37*. To confirm that inactivation of these genes led to mtDNA depletion, we quantified the relative mtDNA copy number of worms submitted to the same RNAi without any EtBr exposure. As shown on Fig. 3, all the animals treated by RNAi, which led to more than 85% EtBr sensitivity, presented obvious mtDNA depletion (10-40% of normal mtDNA content) except *immt-1*. None of these gene silencing resulted in a specific abnormal developmental phenotype (data not shown). To ascertain that the L3 arrest phenotype was due to specific inactivation of the targeted genes, we quantified by reverse transcriptase-PCR (RT-PCR) the expression of most of these genes during the RNAi experiments without EtBr exposure and consistently observed silencing of all genes (Fig. 4). These results therefore suggest that the proteins encoded by these genes are closely related to mtDNA replication.

A second group of genes was found for which inactivation did not result in any EtBr sensitivity (44 to 74% L3 arrest). Among them, *mel-32*, *clpx* (*D2030.2)*, *pyr-1*, *hsp-6* and *ant1-2* encode proteins homologous of human proteins of the core nucleoid whereas *dao-3*, *kat-1* and *immt-2* are orthologs of the class II or III. Moreover, silencing of these genes does not result in major modification of the mtDNA copy number (Fig. 3). The same results were obtained with the inactivation of *irk-1* and *C34B7.2*, two genes encoding proteins of unrelated mitochondrial function. *irk-1* encodes a protein associated with the outer mitochondrial membrane homologous to human *LRRK2* protein whom mutations result in Parkinson disease-8 and *C34B7.2* encodes a putative cytosolic phosphoinositide phosphatase. Thus, whereas the human counterparts of the second group of genes are closely related to mtDNA,

at least in human, they seem not to be involved in mtDNA copy number control in *C. elegans.*

Discussion

We have designed an efficient screening method for the identification of genes involved in the mitochondrial genome stability in *C. elegans*. Indeed, by combining RNAi of *polg-1* and *mtss-1* genes already known to be involved in mtDNA maintenance and EtBr exposure of worms, we were able to rapidly reveal a L3 developmental arrest associated with a severe decrease in mtDNA copy number. It has been shown that there is no mtDNA replication during the early larval stages and that transition from L3 to adulthood is associated with an important increase of mtDNA replication [27, 28]. Therefore we can hypothesize that EtBr exposure drastically inhibited replication of the low mtDNA content resulting from *polg-1* or *mtss-1* silencing. This developmental phenotype seems to be specific as silencing of other genes encoding either mitochondrial proteins not involved in mtDNA maintenance (C29H12.1, lrk-1) or non-mitochondrial proteins (pmp-4, C34B7.2) did not result in a L3 arrest and/or mtDNA depletion. Interestingly, with a notable exception (immt-1), only mtDNA quantitative anomalies were observed. This abnormal and specific phenotype is obvious after three days and therefore allows a very rapid screen that can be further used for large-scale screening in *C. elegans*.

We extended this screen to *C. elegans* genes encoding proteins homologous to human nucleoid proteins. The function of some of these proteins is clearly related to mtDNA stability. Indeed, the mitochondrial RNA polymerase, DNA polymerase, single strand DNA binding protein, Twinkle helicase or the transcription factor TFAM have been shown to directly interact with mtDNA in several species. We applied our screen to the *C. elegans* genes orthologous to human nucleoid encoding protein genes. This clearly showed us that inactivation of genes involved in mtDNA replication or transcription rapidly result in L3 developmental arrest and mtDNA depletion confirming the efficiency of our screen. However, other proteins identified as true components of the human nucleoid (core nucleoid) do not display obvious function related to mtDNA maintenance. This is the case for *SHMT2*, *CPS1* or *CLPX* for example. *SHMT2* (serine hydroxymethyltransferase) is primarily responsible for glycine synthesis, *CPS1* (carbamoyl-phosphate synthetase 1) catalyzes the first committed step of the hepatic urea cycle and *CLPX* gene encodes a ClpX caseinolytic protease X homolog with no well-defined function. Whether these proteins are bifunctional proteins with a second function directly related to mtDNA maintenance or are structural components of the nucleoid with no direct function in mtDNA replication, transcription or translation is still under debate. Silencing of these genes by RNAi combined with EtBr exposure resulted in a normal mtDNA content, or an increased mtDNA content which can be the result of a general mitochondrial upregulation, and a L3 arrest level similar to that observed in control animals

suggesting no functional interaction with the mitochondrial genome.

Furthermore, our screen distinguishes five new genes involved in mtDNA maintenance in a pluricellular organism, *atad-3*, *phi-37*, *dnj-10*, *immt-1* and *Y105E8A.23*.

ATAD3 is an AAA-domain protein, a class of proteins that is known to have important roles in DNA and RNA transactions. In human, *ATAD3* was reported to be an intrinsic mitochondrial membrane protein embedded in the inner membrane leaving its AAA-domain directed towards the matrix [43, 46]. In *C. elegans*, *atad-3*, the homolog of vertebrate *ATAD3*, has been shown to be important for larval development and is required for proper organ function suggesting a crucial role of this protein for the up regulation of mitochondrial activity during the progression through larval stages [47]. Moreover, atad-3 *(RNAi)* animals showed drastic reduction of complex I and citrate synthase (CS) activities. The severe mtDNA depletion we observed after *atad-3* silencing could explain this respiratory chain deficiency. Whether CS decrease is primary related to *atad-3* suppression or secondary to mtDNA depletion has still to be demonstrated. It should be noted that CS is a class III protein of the nucleoid and that its decreased activity in *atad-3 (RNAi)* animals could therefore result from defective nucleoid assembly. Interestingly, in human and in flies it was shown that the ATAD3A protein does not bind mtDNA but rather participates in the connection between the inner and outer mitochondrial membranes regulating the mitochondrial dynamics [48]. Interactions between the mitochondrial inner and outer membranes control a number of central mitochondrial functions such as channeling of metabolites, protein transport, coordinated fusion and fission, and mitochondrial DNA inheritance. The relationship between mitochondrial network and mtDNA maintenance has been already demonstrated as mutations in *OPA1*, encoding a dynamin-related GTPase and being mainly involved in the mitochondrial network organization [49] results in multiple mtDNA deletions [7]. Our results strengthen the idea that ATAD3A, through interactions with proteins of the inner membrane, directly contributes to mtDNA maintenance within nucleoids.

phi-37 encodes the alpha subunit of mitochondrial ATP synthase and the human ATP5A protein is a class III nucleoid protein. It is hypothesized that class III proteins were adventitiously crosslinked to other nucleoid proteins [43]. Nevertheless, suppression of *phi-37* leads to highly sensitive worms to EtBr and mtDNA depletion. Whether this protein plays a direct role on mtDNA stability in addition to its role in the mitochondrial ATP synthase is very interesting and deserves more experiments. It can be also hypothesized that ATP synthase deficiency due to *phi-37* silencing could induce cristae modifications and consequently avoid mtDNA attachment to the inner mitochondrial membrane resulting in a secondary mtDNA depletion [50-52].

dnj-10 is homologous to human *DNAJA3*, also known as Tid1. A crucial role of

DNAJA3 for mitochondrial biogenesis has been demonstrated in mice where a progressive RC deficiency and decreased copy number of mtDNA were reported in cardiomyocytes lacking *DNAJA3* [53]. Hsp70 and PolG have been identified as interactors of DnaJ3 suggesting that DnaJ3, through its chaperone activity on PolG folding, thereby controls mtDNA replication. *DNAJA3* function seems very well conserved through evolution since the invalidation of its homolog in yeast (Mdj1) that also interacts with the yeast mitochondrial DNA polymerase, causes mtDNA loss. Our results show that *dnj-10* in *C. elegans* is also involved in mtDNA copy number control and could thus be a functional homolog of human *DNAJA3*.

Mitofilin (IMMT) is a protein anchored to the mitochondrial inner membrane, with a small N-terminal domain protruding in the mitochondrial matrix [54]. A role in protein import related to maintenance of mitochondrial structure was suggested as mitofilin helps to regulate mitochondrial morphology [55]. Recently, mitofilin has been implicated in the maintenance of the mtDNA integrity as depletion of mitofilin in human cultured cells causes accumulation of mtDNA damage [56]. In *C. elegans* two genes encode the *IMMT* homolog, *immt-1* and *immt-2*. Inactivation of *immt-1* only, the closest homolog of *IMMT*, leads to EtBr sensitivity but not to mtDNA depletion (Fig. 3).

Y105E8A.23 encodes a protein highly homologous to the mitochondrial RNA polymerase (POLRMT). A direct relationship between RNA polymerase and mtDNA copy number has never been observed in animals. Only, the yeast mitochondrial RNA polymerase (*RPO41*) has been involved in the mitochondrial genome stability. The amino-terminal extension of the yeast mtRNA polymerase is required for a mtDNA maintenance function that is separable from the known RNA polymerization activity of the enzyme [57]. Our study adds new evidence of a role of the mitochondrial RNA polymerase on mtDNA stability in a pluricellular organism.

Finally, *C. elegans* genome contains five genes encoding ADP/ATP translocators. We found that only *ant-1.2* (RNAi) worms resulted in a phenotype comparable to control animals. Interestingly, the *ant-1.2* gene is the most divergent *C. elegans ant* gene. On the contrary, down expression of the *C47E12.2* gene, more distantly related to *ant* genes, leads to EtBr sensitivity and mtDNA depletion. The function of ANT proteins is not confined to exchanging ADP/ATP between mitochondria and cytosol. Human ANT1 also contributes to programmed cell death [58] and mtDNA stability as a constituent of the nucleoid [3, 4]. Human ANT2 has been shown to also maintain ATP entry into mitochondria even when oxidative phosphorylation is impaired [59]. These results highlight the differential contribution of these *C. elegans* ANT proteins on mtDNA maintenance.

In conclusion, our work allowed to develop a rapid and efficient screening in *C.*

elegans for genes possibly involved in mtDNA maintenance. By applying this screen to genes homologous to human genes encoding proteins of the nucleoid, we identified new genes (*Y105E8A.23*, *dnj-10*, *atad-3* and *phi-37*) that clearly show a direct control on mtDNA copy number. The human orthologs of these four genes (*POLRMT*, *DNAJA3*, *ATAD3* and *ATP5A1*) should therefore be considered as candidate genes for patients with quantitative mtDNA anomalies. These results will now allow us to systematically invalidate all genes of the *C. elegans* genome with the aim to identify new components of the mitochondrial genome copy number control. It should be hypothesize that false positive will be obtained as it is the case for *immt-1*, the counterpart of fast high throughput screening being of course the risk of false positive or negative results. Obviously several of these genes would have been already identified as such but we hope to find new genes involved in mtDNA maintenance, to identify a yet unknown function of already described genes with no relation to any mitochondrial function or to identify transient nucleoid components in a temporal and/or spatial manner that could play important function on the stability and maintenance of mtDNA. This reservoir of new genes will represent a helpful tool and knowledge for mitochondrial diseases as they would be considered as candidate genes for patients.

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Materials and Methods

Strains and growth conditions

The *C. elegans* wild type strain N2 Bristol and *unc-119 (ed3)III* were used in this work. The strains were maintained at 20℃ on NGM plates seede d with OP50 *E. coli* strain.

Generation of transgenic worms

The entire *mtss-1* gene with the promoter region or the *mtss-1* coding sequence without stop codon were PCR amplified using mtss-1UP or mtss-1ATG as forward primers and mtss-1DW as reverse primer (Table 2). The amplification product of the *mtss-1* coding sequence was cloned into the pPD136.61 vector in fusion with the GFP sequence. The resulting plasmid encoded MTSS1-1::GFP under the control of the *myo-3* promoter. The entire *mtss-1* gene containing the promoter sequence was fused in frame with the GFP coding sequence by using the PCR-fusion method [60] and cloned into the pGEMT-easy plasmid. Both plasmids and the plasmid pDPMM016b carrying an *unc119(+)* gene, were linearized and used to perform microparticle bombardments of the *unc-119 (ed3)* strain using the Bio-Rad Biolistic PDS-1000/HE (Bio-Rad laboratories) as described [61]. Following bombardment, worms were allowed to recover at 20℃ and after 14 days, plates were examined for animals with wild type motility. Confirmation of the presence of the *mtss-1::gfp* transgenes was made by PCR. For each construct two independent transgenic worm lines were generated.

RNA interference and EtBr assay

The RNAi experiments were performed using the feeding procedure described by Kamath and Ahringer (2003) with slight modifications. Feeding RNAi clones were purchased from the Ahringer RNAi library (Geneservice Limited) and sequenced. A single colony of HT115 (DE3) bacteria of interest (RNase III-deficient *E. coli* strain, carrying IPTG-inducible T7-RNA polymerase) was first grown overnight at 37^{o}C in LB-ampicillin. The bacteria were then seeded onto NGM plates with 1 mM IPTG, 25 µg/ml carbenicillin and 50 µg/ml ampicillin and incubated at room temperature in a dark container at room temperature for 48 h to allow the expression of the double-stranded RNA (dsRNA). Worms feeding on HT115 bacteria carrying the empty vector (L4440) were used as controls in all the experiments.

Synchronized L1-stage N2 worms were placed onto NGM plates seeded with bacteria expressing the dsRNA of interest and were incubated for 72 h at 20^{o}C. Four adult worms were independently picked up and transferred to fresh RNAi plates containing or not different concentrations of ethidium bromide (EtBr). Worms were allowed to lay 80 to 100 eggs before being removed. Eggs were immediately counted and the F1 progeny produced was analyzed after 3 and 4 days. At day 4, evaluation of the F1 progeny arrested at the L3 stage was

compared to the number of adults on the same plate. The phenotype was scored as sensitive to RNAi and EtBr if more than 80% of worms were arrested at the L3-stage on plates containing 50 μg/ml of EtBr. A gene was considered as positive for a given phenotype if the same result was observed in at least two independent feeding experiments.

The specificity and efficiency of RNAi inactivation of each gene were checked by semi-quantitative RT-PCR analyses. Total RNA was isolated from N2 animals fed on RNAi bacteria using Trizol reagent (Invitrogen). Total RNA were treated with DNaseI, then subjected to cDNA synthesis using the reverse transcriptase SuperScript VILO™ (Invitrogen) and PCR amplified. The primers used for each gene are listed in Table 2. The amplification of a 426 bp fragment of the *ama-1* cDNA was used as internal control.

Quantification of mtDNA and investigation of mtDNA integrity

Extraction of total genomic and mitochondrial DNA was performed using the NucleoSpin Tissue extraction kit (Macherey-Nagel) from 10 F1-adult worms having laid all their eggs (day 9) after RNAi treatment or from control adults at the same stage. The mitochondrial *cytb* and the nuclear *K01H12.2* genes were individually amplified by real-time PCR using primers cytb-f/cytb-r and K01H12.2-f and K01H12.2-r (Table 2) as previously reported [37]. The ratio of mtDNA copy number to nuclear DNA was used as a measure of mtDNA content in each specimen. Each Q-PCR measurement has been made in triplicate in at least two independent RNAi experiment. The integrity of mtDNA was checked by long-range PCR (Expand Long Template, Roche) using a pair of primers distant of 8561 bp (CE3 and CE4, Table 2).

Legends to Figures

Figure 1. *polg-1* (RNAi) and *mtss-1* (RNAi) lead to mtDNA depletion. **A**, phenotype of *polg-1* (RNAi) and *mtss-1* (RNAi) F1 adult worms. **B**, mtDNA copy number of control (L4440), *polg-1* (RNAi), *mtss-1* (RNAi) and control adults treated from the L4 stage with 125 µg/ml EtBr (EtBr). The relative ratio of mtDNA copy number per adult is represented. **C**, Long-range PCR of the mtDNA extracted from *polg-1* (RNAi) and L4440 control animals (C). M: molecular weight marker. **D**, *polg-1* and *ama-1* mRNA levels in N2 animals grown on bacteria expressing either *polg-1* dsRNA or containing the empty L4440 RNAi vector (C) during larval development and adulthood. M: molecular weight. **E**, Expression of MTSS-1::GFP fusion protein in adult worms. MTSS-1::GFP was expressed either under its own promoter (par-MTSS-1::GFP, (1) Normaski and (*2*) fluorescence) or under the body-wall muscle specific *myo-3* promoter (myo-3-MTSS-1::GFP, (*3*). RNAi of *mtss-1* gene in *myo3*-MTSS-1::GFP transgenic animals (*5*) compared to control RNAi (4).

Figure 2. *polg-1* (RNAi) and *mtss-1* (RNAi) mutants are hypersensitive to the EtBr. **A**, L3 larval development arrest after *polg-1* (RNAi) and *mtss-1* (RNAi) in the presence of 40 µg/ml of EtBr compared to control animals (L4440). **B**, Percentage of L3 larval arrest of *polg-1* (RNAi) (square), *mtss-1* (RNAi) (triangle) and control animals (L4440) (circle) grown with increasing concentration of EtBr. Each point corresponds to the analysis of *n*> 80 animals in two independent experiments. **C**, Percentage of L3 arrest of worms submitted to RNAi of *polg-1*, *mtss-1*, *rrt-2* and *pmp-4* genes and 50 µg/ml EtBr exposure. L4440: control RNAi.

Figure 3. Correlation between L3 arrest and mtDNA copy number. mtDNA content of adult worms after 9 days of RNAi of various genes (black bars). Values are presented as ratio of mtDNA versus nuclear DNA relative to the control (L4440 RNAi). The error bars represent the SD. Percentage of L3 arrested animals after RNAi and 50 µg/ml EtBr exposure (gray bars). Experiments were performed in at least two independent RNAi for each gene.

Figure 4. Efficiency and specificity of RNAi. The expression level of various genes was estimated by semiquantitative RT-PCR after RNAi of the corresponding genes and in control (L4440). *ama-1* transcript level was used as internal control. M: molecular weight

Table 1. List of *C. elegans* genes homologous to human nucleoid genes. The three classes correspond to the nucleoid class of proteins described in Bogenhagen *et al*., 2008. Class I: proteins isolated in both native and crosslinked nucleoid preparations, class II: proteins observed in native nucleoids but not found crosslinked to mtDNA and class III: proteins

identified in crosslinked nucleoids but not found in native nucleoids. The two last columns show the Blast E-values between *C. elegans* and human genes.

Table 2
Oligonucleotides used in this study.

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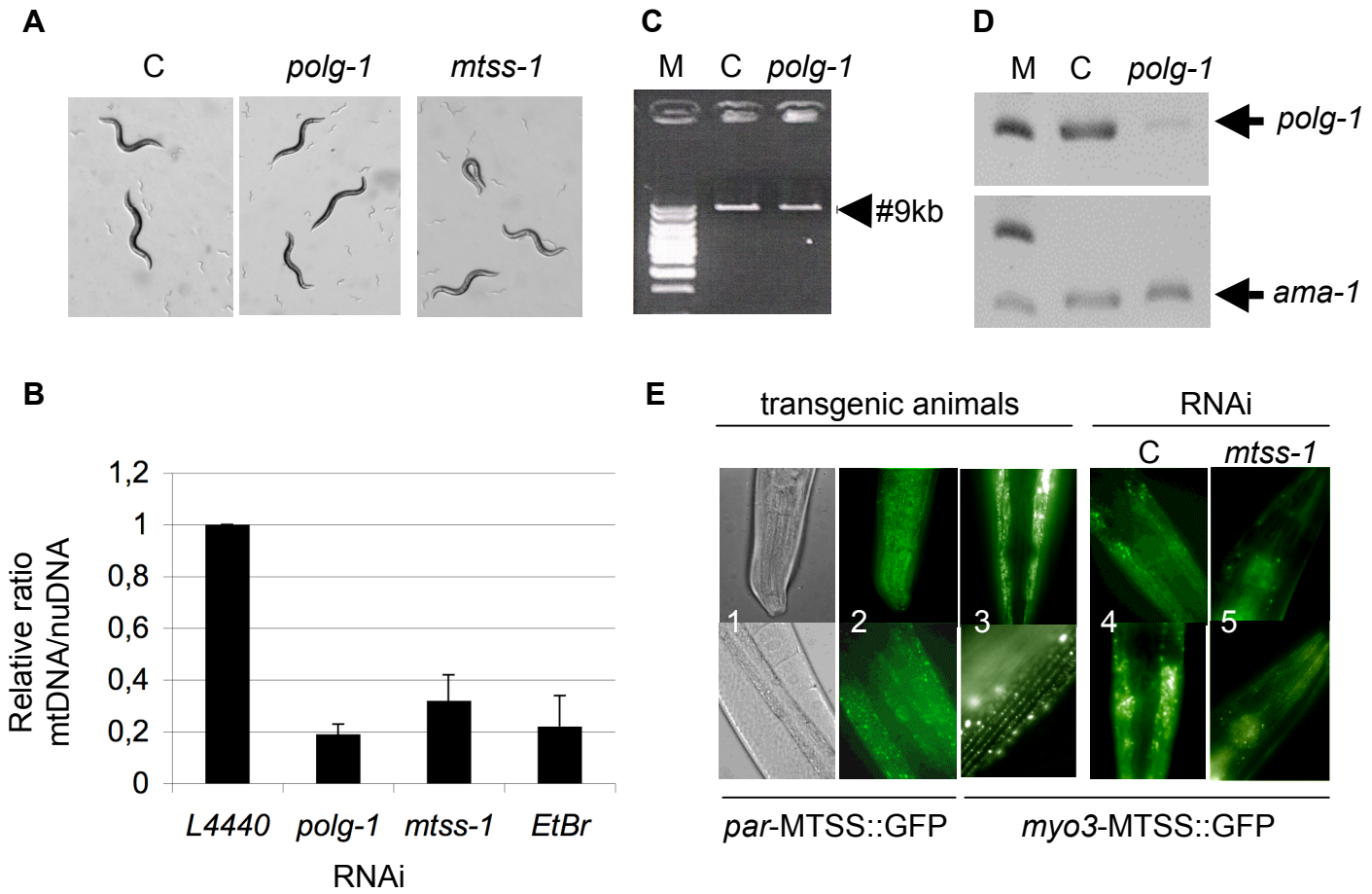

Figure 1

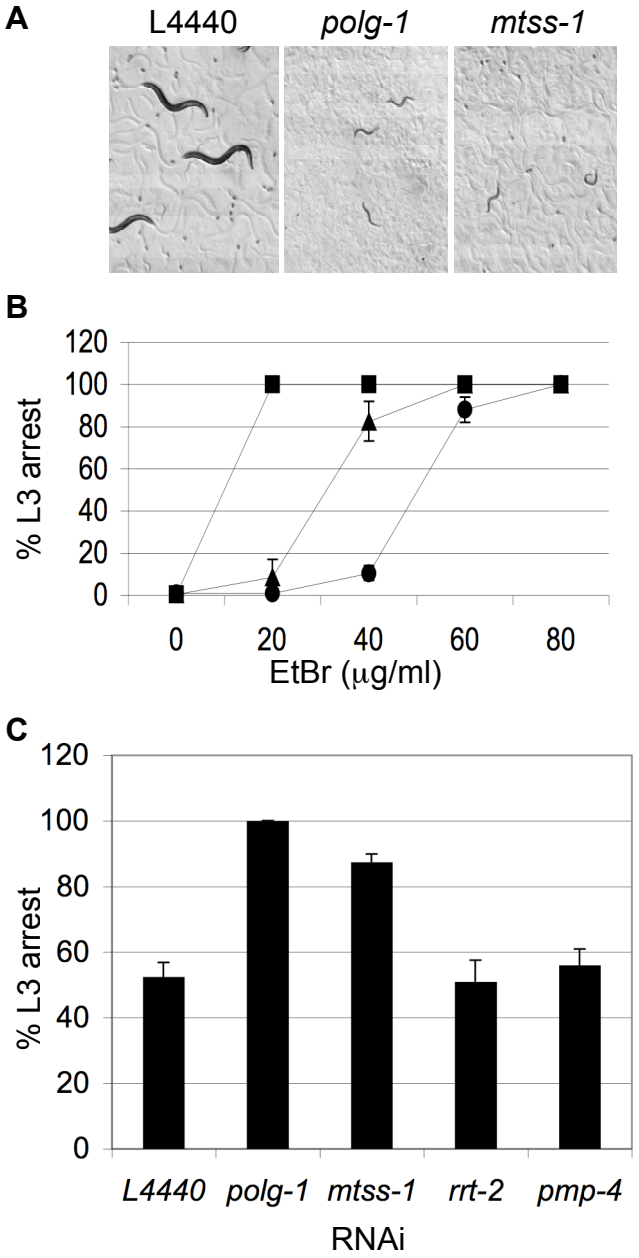

Figure 2

Figure 3

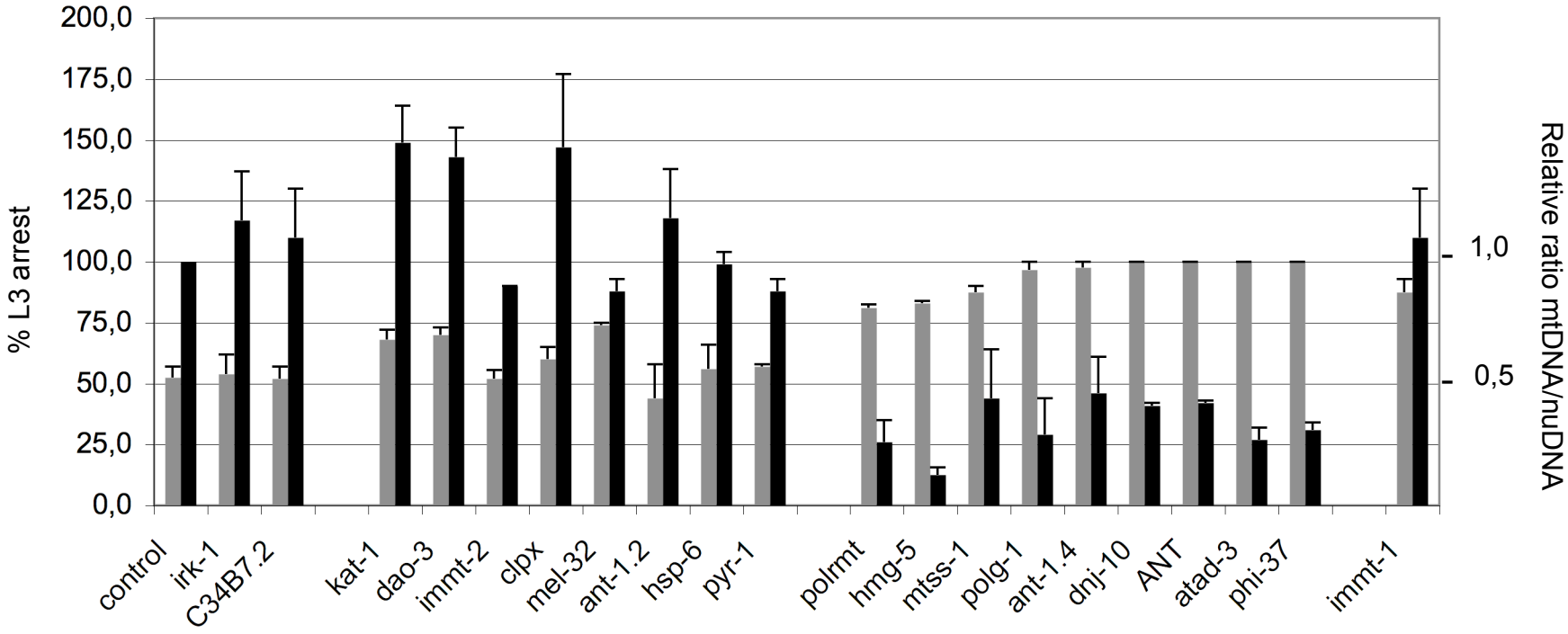

Figure 3

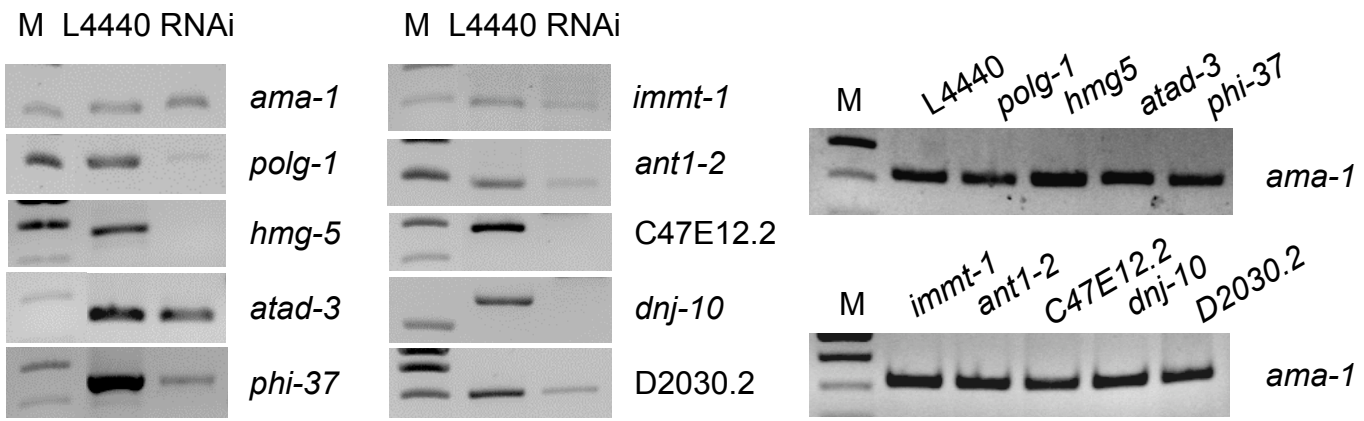

Figure 4

Table 1

Nucleoid class	Human gene	C. elegans gene	C. elegans main gene	C. elegans description	Blast E-value	% length
I	TFAM	F45E4.9	hmg-5	HMG box-containing protein	4.0e-18	91.2%
I	SSBP1	PAR2.1	mtss-1	Single-stranded DNA-binding protein	1.2e-09	65.9%
I	POLG	Y57A10A.15	polg-1	ortholog of human mitochondrial DNA polymerase gamma	7.6e-102	67.3%
I	POLRMT	Y105E8A.23		no description	1.2e-112	54.8%
I	PEO1	F46G11.1		orthologous to the human mitochondrial DNA helicase twinkle	9.7e-56	80.3%
I	CLPX	D2030.2		Putative ATP-dependent Clp-type protease (AAA+ ATPase superfamily)	1.2e-103	91.8%
I	DNAJA3	F22B7.5	dnj-10	protein containing a DnaJ ('J') domain that is predicted to be mitochondrial.	5.6e-83	83.8%
I	ANT2/3	C47E12.2		Mitochondrial ADP/ATP carrier proteins	9.9e-77	94.4%
I	ANT2/3	W02D3.6	ant-1.2	Mitochondrial ADP/ATP carrier proteins	8.49e-103	96.0%
I	ANT2/3	T01B11.4	ant-1.4	Mitochondrial ADP/ATP carrier proteins	1.0e-106	92.0%
I	SHMT2	C05D11.11	mel-32	Glycine/serine hydroxymethyltransferase	5.4e-156	91.9%
I	HSPA1	C37H5.8	hsp-6	mitochondrial-specific chaperone that is a member of the DnaK/Hsp70 superfamily	1.5e-261	97.0%
I	CPS1	D2085.1	pyr-1	orthologous to the human gene CPS1 carbamyl phosphate synthetase	0	65.9%
II	ATADA3	F54B3.3	atad-3	AAA+-type ATPase	9.79e-173	96.3%
II	IMMT	T14G11.3	immt-1	Mitochondrial inner membrane protein (mitofilin)	1.8e-68	86.5%
II	IMMT	W06H3.1	immt-2	Mitochondrial inner membrane protein (mitofilin)	8.7e-32	86.9%
III	MTHFD2	K07E3.3	dao-3	C1-tetrahydrofolate synthase	8.7e-78	95.4%
III	ACAT1	T02G5.8	kat-1	homolog of the human gene ACAT1 Acetyl-CoA acetyltransferase	4.3e-115	94.6%
III	ATP5A1	H28O16.1	phi-37	F0F1-type ATP synthase, alpha subunit	2.1e-225	93.5%

Table 1

Table 2

	target genes	primers	PCR length
mtss1	mtss-1UP	GAAGTTTTCTTGTGAAGAAGC	
	mtss-11ATG	GGAAGATCTTAGAAAATGCTTCGTTCACTT	
	mtss-1DW	CGGGATATCGGAAACAGTTATGTTTGG	
RT-PCR	ama-1-f (95-112)	CAGTGGCTCATGTCGAGT	
	ama-1-r (559-577)	CGACCTTCTTTCCATCAT	482 bp
	ant1-2-f (46-65)	CTCGCCTCCGGAGGCACTGC	
	ant1-2-r (606-627)	GCCCGTCAGTTGAGTACAATG	581 bp
	atad-3-f (291-311)	GGCCAATATGAAATCAGAGCA	
	atad-3-r (626-647)	CGGTTCTCTTCTTCGTGAAGC	356 bp
	dnj-10-f (692-711)	GTAATCGATGCAGAGGAAGT	
	dnj-10-r (1142-1162)	CGCAGCCCAAGCCAACATAA	470 bp
	C47E12.2-f (251-271)	GGAGAGGAAACATGACAAATG	
	C47E12.2-r (717-737)	GTGTCCCAAGGGTAGGTTAA	486 bp
	immt-1-f (385-404)	CCAAGAGAGCCCACACATGT	
	immt-1-r (814-834)	GATCTCATCAAGCTGATGAG	449 bp
	D2030.2-f (663-682)	GCAGCAGCAGAGTAATAATC	
	D2030.2-r (1164-1184)	CCAGAAGACGTACCAAATCC	521 bp
	hmg-5-f (52-71)	CGTGCTTCTGTCGCAGCTTC	
	hmg-5-r (563-583)	CCCATTTCTGGAGGACGACA	531 bp
	polg-1-f (19-39)	ATGCAAATTTTCCACGTATCC	
	polg-1-r (619-638)	CCCGACATCTGGCTCGATC	619 bp
	phi-37-f (53-73)	ATGCCGGAATCGCCACCACCG	
	phi-37-r (507-528)	GATGGTGTCAATGGCAATGGC	475 bp
q-PCR	cyb-f (158-177)	CGCCCGATAGGTTAATAGCA	
	cyb-r (234-253)	TGGCCCCATTAAAATGAAAA	95 bp
	K01H12.2-f (502-522)	CGTGAATTCAAAGGTCTGGCT	
	K01H12.2-r (602-622)	AGTAAGCGGCACGGTAGATGA	120 bp
deletion	CE3 (1840-1860)	GAGCGTCATTTATTGGGAAG	
	CE4 (10529-10550)	CACAAAGGTCGACATATCAAC	8710 bp